P153 AND P156 ANTIGENS FOR THE IMMUNODIAGNOSIS OF CANINE AND HUMAN EHRLICHIOSES AND USES THEREOF

Cross-reference to Related Application

This application claims benefit of provisional patent application U.S. Serial No. 60/423,573, filed November 4, 2002, now abandoned.

Federal Funding Legend

This invention was produced in part using funds from the Federal government under Grant No. AI31431 from the National Institute of Allergy and Infectious Diseases. Accordingly, the Federal government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of molecular and immunodiagnostics. More specifically, the present invention relates to species-specific immunoreactive protein orthologs (~200 kDa) from *Ehrlichia canis* and *Ehrlichia chaffeensis* that are useful for species-specific diagnosis of canine ehrlichiosis and human monocytotropic ehrlichiosis.

Description of the Related Art

Canine monocytic ehrlichiosis is a potentially fatal tick-borne disease of dogs with worldwide distribution caused primarily by the rickettsial agent, *Ehrlichia canis* (Huxsoll et al., 1970). *E. canis* is an obligately intracellular bacterium that exhibits tropism for monocytes and macrophages (Nyindo et al., 1971), and establishes persistent infections in the vertebrate host (Harrus et al., 1998). The disease is characterized by three stages: the acute stage which lasts 2 to 4 weeks; the subclinical stage, in which dogs can remain persistently infected for years, but do not exhibit clinical signs, followed by the chronic phase, where in many dogs

the disease becomes progressively worse due to bone marrow hypoplasia and the prognosis less favorable (Troy et al., 1990).

Ehrlichia canis infects and causes ehrlichiosis in animals belonging to the family Canidae. Canine ehrlichiosis consists of an acute and a chronic phase. The acute phase is characterized by fever, serous nasal and ocular discharges, anorexia, depression, and loss of weight. The chronic phase is characterized by severe pancytopenia, epistaxis, hematuria, blood in feces in addition to more severe clinical signs of the acute disease. If treated early during the course of the disease, dogs respond well to doxycycline. However, chronically infected dogs do not respond well to the antibiotic. Therefore, early diagnosis is very important for treating canine ehrlichiosis.

Treating the disease in the acute phase is important for the best prognosis. Hematologic abnormalities such as leukopenia and thrombocytopenia often provide useful evidence of canine ehrlichiosis and are important factors in the initial diagnosis (Troy et al., 1990). However, diagnosis is made difficult because the clinical presentation of canine ehrlichiosis is non-specific.

Diagnosis of canine ehrlichiosis by serologic methods such as the indirect fluorescent-antibody (IFA) test has become the standard method due to its simplicity, reliability and cost effectiveness (Troy et al., 1990). However, shortcomings of the indirect fluorescent-antibody test include the inability to make a species-specific diagnosis due to antigenic cross reactivity with other closely related *Ehrlichia* species that infect dogs (*E. chaffeensis, E. ewingii, Anaplasma phagocytophilum,* and *A. platys*). Subjective interpretations may also result in false-negative results, or false-positives caused by cross-reactive antigens. Other diagnostic methods such as polymerase chain reaction (PCR) have been developed for specific detection of *E. canis,* and were reported to be more sensitive than cell culture isolation, but this method requires specialized training and expensive equipment (McBride et al., 1996). Isolation of the organism is time consuming, and only a few laboratories have been consistently successful with this method. Furthermore, additional tests characterizing the isolate are required for defining a specific etiology using this method.

Serologically cross-reactive antigens shared between *E. canis* and *E. chaffeensis* have been reported. Some of the major serologically cross-reactive proteins exhibit molecular masses of 28-30-kDa (Chen et al., 1997; Rikihisa et al., 1994), and it is now known that these proteins are encoded by homologous multigene families (Ohashi et al., 1998a, b). There are 22 and 25 homologous, but nonidentical, *p28* genes that have been identified and sequenced in *E. chaffeensis* and *E. canis,* respectively. Similar intraspecies and interspecies strain homology was observed between the P28 proteins of *E. canis* and *E. chaffeensis,* explaining the serologic cross reactivity of these proteins (McBride et al., 1999).

A recent report demonstrated that the rP28 protein from *E. chaffeensis* was an insensitive tool in diagnosing cases of human monocytotrophic ehrlichiosis (HME) (Yu et al., 1999a). The underlying reason appears to be the variability of the P28 protein among different strains of *E. chaffeensis* (Yu et al., 1999b). Conversely, the P28 genes identified in *E. canis* are conserved among geographically dispersed strains, and the *E. canis* rP28 has proven to be useful for diagnosis of canine ehrlichiosis (McBride et al., 1999; Ohashi 1998a). Other homologous immunoreactive proteins

including the glycoproteins in *E. canis* (gp140) and *E. chaffeensis* (gp120) have been cloned (Yu et al., 1997, 2000). Reactivity of the rgp120 of *E. chaffeensis* has correlated well with the indirect fluorescent-antibody for serodiagnosis of human monocytotropic ehrlichioisis, and preliminary studies with the rgp140 of *E. canis* suggest that it may be a sensitive and reliable immunodiagnostic antigen (Yu et al., 1999a, 2000).

The prior art is deficient in specific antigens for serologic and molecular diagnostics for *E. canis* and *E. chaffeensis* as well as methods for such use. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

A strongly immunoreactive 43 kD protein (p43) of *Ehrlichia canis* has been identified (U. S. Pat. No. 6,355,777). As an immunodiagnostic antigen, the p43 had a 96% accuracy as compared with the indirect fluorescent-antibody test and provided

species-specific diagnosis of *E. canis* infections. Further investigation revealed that the *E. canis* p43 represents the N-terminal portion of a protein with a predicted molecular mass of 153 kD, the largest immunoreactive protein described in *Ehrlichia* spp. Analysis of recombinant expressed fragments of the p153 by protein gel electrophoresis demonstrated a larger than predicted molecular mass (~10 to 30%) and presence of carbohydrate glycans on N- and C-terminal fragments, indicating that the p153 is a glycoprotein.

A BLASTn search was performed on the available *E. chaffeensis* genome sequence (95%), and the gene encoding the *p153* ortholog was identified in *E. chaffeensis*. The *E. canis p153* (4263-bp) and *E. chaffeensis p156* (4389-bp) genes had similar chromosomal locations, downstream of the homologous (~87%) deoxyguanosine-triphosphate triphosphohydrolase genes and homologous (~90%) intergenic sequences preceding the open reading frames. Nucleic acid sequence homology (50%) was observed between the glycoprotein genes, supporting previous findings with regard to genetic divergence of the *p43* gene fragment, and the *p153* and *p156* proteins had amino acid similarity of 32%.

A native *E. canis* protein with a molecular mass of 200 kD reacted with antisera produced against the N-terminal region (p43) of the p153, suggesting that the native protein was post-translationally modified. Similarly, a recombinant protein comprising the N-terminal region of *E. chaffeensis* p156 migrated larger than predicted (~200 kD), and carbohydrate was detected on the recombinant protein. A major immunoreactive epitope was identified in this N-terminal fragment. The chromosomal location, amino acid homology, and biophysical properties support the conclusion that the p153 and p156 glycoproteins (designated gp200s) are species-specific immunoreactive orthologs.

Major immunoreactive epitopes has been identified in the N- (P43) and C-terminal regions of the *E. canis* p153 and the N-terminal region of the *E. chaffeensis* p156 ortholog that will be useful for serologic diagnostics and vaccines. Furthermore, genes encoding these proteins are species-specific and will be useful for the development of molecular-based diagnostics.

Other and further aspects, features, and advantages of the present invention will be apparent from the following

description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figures 1A and **1B** show Lipman-Pearson amino acid alignment of the *E. chaffeensis* p156 (top line) and the *E. canis* p153 (bottom line) protein orthologs. Amino acid identities, conserved (:) and semiconserved (.) substitutions are shown in the center.

Figures 2A and 2B show expression of recombinant protein fragments from the *E. canis* p153 **(A)** and *E. chaffeensis* **(B)** and detection with anti-V5 antibody. *E. canis* p153, lane 1, N-terminal fragment (1107-bp, nt-1-1107), lane 2, internal fragment (910-bp, nt-1080-1990), lane 3, internal fragment (1000-bp, nt-1950-2950), and lane 4, C-terminal fragment (1280-bp, nt-2940-4220). *E. chaffeensis* p156, lane 1, N-terminal fragment (1545-bp, nt-125-1675), lane 2, internal fragment (1365-bp, nt-1685-3050), and lane 3, C-terminal (1365-bp, nt-2950-4315).

Figure 3A shows Western immunoblot of *E. canis* p153 recombinant fragments. Lane 1, N-terminal fragment (1107-bp, nt-1-1107), lane 2, internal fragment (910-bp, nt-1080-1990), lane 3, internal fragment (1000-bp, nt-1950-2950), and lane 4, C-terminal fragment (1280-bp, nt-2940-4220).

Figure 3B shows carbohydrate detection on corresponding purified recombinant fragments of the *E. canis* p153 expressed in *E. coli* using the pRSET expression vector. Glycans

attached to the recombinant proteins were oxidized, labeled with biotin and detected with streptavidin-alkaline phosphatase.

Figure 4A shows Western blot of the *E. chaffeensis* p156 recombinant fragments (lanes 1-3) with human (left panel) and dog serum (right panel). Lane 1, *E. chaffeensis* p156 N-terminal fragment (1545-bp, nt-125-1675), lane 2, internal fragment (1365-bp, nt-1685-3050), and lane 3, C-terminal (1365-bp, nt-2950-4315). Expressed recombinant proteins represent ~95% of the *E. chaffeensis* p156.

Figure 4B shows carbohydrate detection of the three corresponding recombinant *E. chaffeensis* p156 proteins (Lanes 1-3).

Figure 5 shows Western blot demonstrating the proteins in *E. canis* whole cell lysate with polyclonal antisera from an *E. canis* infected dog (lane 1) and anti-recombinant p43 (gp200) (lane 2) and anti-recombinant gp140 (lane 3) polyclonal rabbit serum.

DETAILED DESCRIPTION OF THE INVENTION

The *E. canis p43* gene sequence was previously reported as 1173-bp (U. S. Pat. No. 6,355,777), but further analysis revealed a DNA sequencing error resulting in an artificial termination codon and a truncated gene sequence. Using the primer-adaptor gene walking method, an additional 4.5-kbp sequence downstream of the 2.4-kbp in the original *p43* clone was determined. The incomplete *p43* gene sequence was completed revealing an open reading frame of 4263-bp, which encoded a protein with a predicted molecular mass of 153 kD (designated p153). Upstream of the *p153* gene there is an open reading frame encoding a deoxyguanosine-triphosphate triphosphohydrolase and an intergenic noncoding region preceding the *p153* gene that have high nucleic acid homology (87% and 90%, respectively) between *E. canis* and *E. chaffeensis.*

A BLASTn search of the *E. chaffeensis* genome sequence with the 2.4-kbp *p43* clone identified a highly homologous nucleic acid sequences. A large open reading frame (4389-bp)

approximately equivalent in size to the *E. canis p153* was found in the same chromosomal location with respect to the upstream homologous coding and intergenic nucleic acid sequences and encoded a protein with a predicted molecular mass of 156 kD (p156). Nucleic acid sequence homology (~50%) was observed between the *E. canis p153* and the *E. chaffeensis p156* genes; however, the proteins exhibited an overall amino acid sequence similarity of 32% (Figure 1).

Gene constructs expressed in *E. coli* representing the *E. chaffeensis* p156 protein (nt-125-1670; nt-1685-3050; nt-2950-4315) and four recombinant fragments of *E. canis* p153 (nt-1-1107 (p43); nt-1080-1990; nt-1950-2950; nt-2940-4220) were expressed in *E. coli* (Figure 2). The *E. canis* N-terminal (nt 1-1107) and C-terminal (nt-2940-4220) recombinant expressed proteins exhibited strong immunoreactivity (Figure 3A). However, only the N-terminal fragment (nt-125-1670) of *E. chaffeensis* p156 was immunoreactive (Figure 4A).

The *E. canis* (nt-1-1107 and nt-2940-4420) and *E. chaffeensis* p156 recombinant proteins fragments (nt-125-1607)

migrated larger than predicted by SDS-PAGE indicating that post translational modification of this fragments had occurred. Subsequently, carbohydrate was detected on the *E. canis* p153 and *E. chaffeensis p156* peptide fragments (Figures 3B and 4B).

Anti-p43 antibody reacted with a native protein of approximately 200 kD in *E. canis* whole cell lysates. Furthermore, this 200 kD protein was also recognized by sera from an *E. canis*-infected dog (Figure 5). A partial gene sequence previously identified as *p43* (N-terminal portion of the p153) assigned GenBank accession number AF252298. The amended sequencing encoding p153 was assigned the GenBank accession number AY156950.

The chromosomal location, amino acid homology, and biophysical properties support the conclusion that the p153 and p156 glycoproteins (designated gp200s) are species-specific immunoreactive orthologs. These proteins have potential uses in vaccine development and can be used as sensitive and reliable serodiagnostic antigens for the diagnosis of *Ehrlichia* infections. This is supported by previous findings that showed the

immunoreactivity and potential use of the *E. canis* p43 as serodiagnostic antigen (U. S. Pat. No. 6,355,777). Reaction with antibodies against p43 had a 100% correlation with samples having an indirect fluorescent-antibody (IFA) titer >40 and did react with several samples with indirect fluorescent-antibody titers of <40. The weak reactivity of several indirect fluorescent-antibody negative samples with the p43 antibodies suggests that p43 protein may be a more sensitive serodiagnostic antigen. The results presented in the present invention indicate that p43 is part of a larger p153 protein in *E. canis.*

The current invention is directed to isolated polynucleotides encoding *Ehrlichia canis* immunoreactive surface protein p153 and *Ehrlichia chaffeensis* p156 protein. Preferably, the isolated polynucleotides encode the proteins with amino acid sequences shown in SEQ ID No: 1 and 2. Alternatively, the DNA may differ in nucleotide sequence due to the degeneracy of the genetic code.

The instant invention also encompasses vectors comprising these isolated polynucleotides and regulatory elements

necessary for expression of the DNA in a cell; isolated and purified p153 and p156 proteins; and antibodies directed against these proteins.

The instant invention is further directed to the use of the p153 and p156 proteins in the preparation of vaccines against canine and human ehrlichioses. In addition, there are provided methods of determining whether a dog or human is infected with an *Ehrlichia* species by determining whether serum from the dog reacts with the p153 or p156 protein. The proteins used may be from recombinant sources, and Western blot analysis may be used to detect the reaction of the serum to the proteins. As reaction with previously isolated *E. canis* p28 protein is also reliable marker of *E. canis* infection, diagnosis may consist of detecting immunoreactivity to the p153 protein, gp140, and the p28 antigens of *Ehrlichia canis.*

The instant invention is also directed to a serodiagnostic kit for determining whether a dog or human is infected with an *Ehrlichia* species. The kit comprises immobilized proteins (p153 or p156) disclosed herein, appropriate dilution buffers for dog serum, anti-dog serum second antibody linked to a reporter molecule, and

appropriate reagents for detection of the reporter molecule. Possible methods of immobilizing the antigens include linkage to membranes or microtiter plates. The reporter molecule may be luciferase, horseradish peroxidase, β-galactosidase, or a fluorescent label.

The instant invention is also directed to a PCR amplification method of determining whether a dog has been infected with an *Ehrlichia* species. DNA is extracted from the blood of a potentially infected dog or human and subjected to PCR amplification with oligonucleotide primers specific for the *E. canis p153* gene or the *E. chaffeensis p156* gene. The resulting PCR amplification products are separated by size by a method such as gel electrophoresis and detection of an appropriately sized product indicates *Ehrlichia* infection.

The instant invention is also directed to a kit for the PCR detection of the *p153* or *p156* gene. The kit comprises reagents for DNA extraction from blood, *p153* or *p156* specific oligonucleotides, and reagents for PCR amplification.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

As used herein, the term "host" is meant to include not only prokaryotes but also eukaryotes such as yeast, plant and animal cells. A recombinant DNA molecule or gene which encodes a protein of the present invention can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art. Prokaryotic hosts may include *E. coli*, *S. tymphimurium*, *Serratia marcescens* and *Bacillus subtilis*.

Eukaryotic hosts include yeasts such as *Pichia pastoris*, mammalian cells and insect cells.

In general, expression vectors containing promoter sequences which facilitate efficient transcription of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth. Methods which are well known to those skilled in the art can be used to construct expression vectors containing appropriate transcriptional and translational control signals. See for example, the techniques described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Press, N.Y.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under

conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced. The conditions include the presence of nucleotides and an inducing agent such as a DNA polymerase and a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and the method used. For example, for diagnostic applications, the oligonucleotide primer typically contains 15-25 or more nucleotides depending on the complexity of the target sequence. Primers with fewer nucleotides may also be used.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the

strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementary with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Characterization of *E. canis* p153 and *E. chaffeensis* p156 Proteins

The *E. canis* p43 protein gene was identified from a Lambda Zap II expression library as previously described (McBride et al., 2001; U. S. Pat. No. 6,355,777). The original 2.4-kb clone consisted of an open reading frame (ORF) encoding a deoxyguanosine-triphosphate triphosphohydrolase gene and a downstream 229-bp intergenic space preceding the truncated *p43* gene fragment. A primer-adapter PCR method was used to

determine the complete sequence of the *p43* open reading frame using *E. canis* genomic DNA (Jake, North Carolina strain) as a template. The amplicons were sequenced directly with primers used for amplification or cloned into TOPO/TA for sequence analysis. The *E. chaffeensis* ortholog (*p156* gene) was identified by performing a BLASTn search of the *E. chaffeensis* genome sequence with the entire *E. canis p43* clone (2.4-kb).

The *E. canis p153* and *E. chaffeensis p156* genes were divided into large fragments (1 to 1.5-kbp), cloned into pUni/V5-His-TOPO Echo donor vector, and recombined with pBAD Thio-E or pRSET Echo acceptor expression vectors. The recombinant proteins were expressed for 4 h after induction with arabinose or IPTG. Glycan detection on expressed recombinant proteins was performed using an immunoblot kit for glycoprotein detection (Bio-Rad) following the membrane labeling protocol. The *E. chaffeensis* recombinant Dsb protein described previously (McBride et al., 2002) was expressed in *E. coli* and used as an ehrlichial negative control protein for glycoprotein detection studies. *E. canis* whole cell lysates were separated by gel electrophoresis using gradient gels (4-12% Bis-Tris, Novagen) and transferred onto pure nitrocellulose

using a semidry transfer unit (Bio-Rad). Immunoblotting was performed as previously described (McBride et al., 2001).

Discussion

The strong immunoreactivity of the clone containing the N-terminal (p43) portion of the *E. canis* p153 led to its initial identification and characterization (McBride et al., 2001). When compared to the results of indirect fluorescent-antibody test for detection of antibodies to *E. canis* in dogs, the p43 exhibited excellent sensitivity and specificity. In addition, the p43 appeared to provide species-specific detection, as anti-recombinant p43 polyclonal antibody did not react with *E. chaffeensis*-infected DH82 cells. The identification of the p153 ortholog in *E. chaffeensis* (p156), which is genetically divergent and has a low degree of amino acid homology, supports previous findings that the p43 protein is a species-specific antigen, and thus would be an excellent species-specific immunodiagnostic antigen. Major linear B cell epitopes are present in the N- (p43) and C-terminal regions of the p153 protein.

The p43 recombinant protein exhibited a larger than predicted molecular mass (~30% or ~10 kD) that was initially unrecognized. Previously reported ehrlichial glycoproteins gp120 and gp140 were 60 to 100% larger than expected. Although the degree of molecular mass shift was much smaller, the p43 protein is a glycoprotein which was confirmed by carbohydrate detection of attached glycans. Consistent with the p43 findings, the expressed *E. chaffeensis* p156 recombinant gene fragments exhibited a larger than expected molecular mass, and carbohydrate was detected on these fragments. Additionally, the C-terminal fragment of the *E. canis* p153 also exhibited larger than predicted molecular mass (~10% or 6kD).

When the *p43* gene was identified, a corresponding native *E. canis* protein from whole cell lysates did not react with anti-p43 antisera. Based on the findings presented here, this discrepancy can be attributed to the fact that the *p43* gene represents an incomplete open reading frame, and it does not encode a 43 kD protein. In addition, the large molecular mass of this protein (>150 kD) requires special attention to gel electrophoresis conditions in order to obtain consistent

identification of this protein by immunoblot. The 200 kD protein in *E. canis* whole cell lysates was strongly immunoreactive with the anti-p43 polyclonal antibody. The molecular mass of this protein is consistent with the predicted mass of the p153 coupled with some glycans contributing to the increased molecular mass. This finding is also consistent with the molecular mass of the *E. chaffeensis* p156 recombinant fragments representing nearly the entire open reading frame.

Glycoproteins of *Ehrlichia* spp. are some of the first such proteins to be characterized in pathogenic bacteria. The ehrlichial glycoproteins discovered to date are consistently and strongly recognized by antibodies in infected patients and animals. These unique surface-exposed immunoreactive proteins have potential in vaccine development, and these proteins may be important components of subunit vaccines.

The following references were cited herein:

Chen, et al., 1997. Western immunoblotting analysis of the antibody responses of patients with human monocytotropic ehrlichiosis to different strains of *Ehrlichia chaffeensis* and *Ehrlichia canis*. Clin. Diagn. Lab. Immunol. 4:731-735.

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Yu, et al., 1997. Cloning and sequencing of the gene for a 120-kDa immunodominant protein of *Ehrlichia chaffeensis.* Gene 184:149-154.

Yu, et al., 1999a. Comparison of *Ehrlichia chaffeensis* recombinant proteins for serologic diagnosis of human monocytotropic ehrlichiosis. J. Clin. Microbiol. 37:2568-2575.

Yu, et al., 1999b. Genetic diversity of the 28-kilodalton outer membrane protein gene in human isolates of *Ehrlichia chaffeensis.* J. Clin. Microbiol. 37:1137-1143.

Yu, et al., 2000. Molecular cloning and characterization of the 120-kilodalton protein gene of *Ehrlichia canis* and application of

the recombinant 120-kilodalton protein for serodiagnosis of canine ehrlichiosis. J. Clin. Microbiol. 38:369-374.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.